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Pleurotus nebrodensis (Basidiomycota), a rare endemic mushroom of Sicily: current and future issues

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Abstract

This paper deals with the basidiomycete *Pleurotus nebrodensis*, one of the rare examples of endemic fungi in Italy and Europe. After clarifying the taxonomy of this taxon, including a comparison with a species from China misidentified as "*Pleurotus nebrodensis*", we describe the characteristics of its natural habitat, and provide a new morphological description and information on its pilot-scale cultivation, current conservation status according to IUCN criteria and conservation strategies. New ITS region sequences were deposited in GenBank. Furthermore, the antibacterial and anti-cancer properties of *P. nebrodensis* are mentioned, making it a potential medicinal mushroom. Finally, a critical analysis, on a phylogenetic basis, of the Italian production of substrates inoculated with "*P. nebrodensis*" strains is also included.

Keywords

Basidiomycete, fungal diversity, medicinal mushroom, mediterranean area

Introduction

The term endemism refers to a species whose occurrence is indigenous and exclusive to limited territories and lacking in the surrounding and distant ones. Endemic species are generally extremely vulnerable to climate change because evolution has led them to

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be formed exactly for that site (Veron et al. 2019). The application of the term endemic for fungi is problematic, although some cases have been demonstrated over time. The study of fungal endemism, especially on islands, is often hampered by insufficient data making it difficult to apply the term endemic and evaluate case studies (Stallman et al. 2022). In the State of Biodiversity in Italy, published in 2005 (Blasi et al. 2005), 56 fungal species are reported as possible endemics.

The environmental characteristics of Sicily and its numerous ecosystems allow for high levels of biodiversity to be found on the island in all groups of organisms. Plant species endemic to Sicily amount to about 15% and include taxa with a punctiform distribution (Di Gristina et al. 2022) while only one fungal species, *Pleurotus nebrodensis* (Inzenga) Quél. (Pleurotaceae), is currently reported as endemic (Ferraro et al. 2022).

Along with *Alessioporus ichnusanus* (Alessio, Galli & Littini) Gelardi, Vizzini & Simonini (Angelini et al. 2021) and *Poronia punctata* (L.) Fr. (Ceci et al. 2021), *P. nebrodensis* is one of the few Italian fungal species currently included on the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species and is in the TOP 50 Mediterranean Island Plants (https://top50.iucn-mpsg.org/species/39). This condition makes *in situ* and *ex situ* conservation strategies necessary.

Among the conservation strategies indicated by Courtecuisse (2001), that of natural habitat conservation appears to be the most effective. Based on International Union for Conservation of Nature (IUCN) criteria, the Extent of Occurrence (EOO) of the *P. nebrodensis* population is less than 100 km^2. The population is severely fragmented and there has been a progressive decline in the number of localities, now fewer than ten (Pasta et al. 2017). The number of mature individuals is <150 with alternating pattern in the fruiting years with an average of *ca.* 70 basidiomata/year. This is mainly due to the increasing number of collectors (professional and amateur), motivated by the high commercial price (50–60 euros per kg) and the remarkable organoleptic properties of this mushroom, despite the absence of a real market for the product. Another negative drawback of the increasing human pressure is the harvesting of young basidiomata, which may seriously affect fruiting of this prized species in the near future (Gargano et al. 2011).

Article 5 of the Madonie Park's Regulation on the Collection of Epigean Mushrooms, issued in 2017, the territory within which all the growth localities of the rare endemic mushroom fall, states that the collection of *P. nebrodensis* in zone A, a wild zone under total protection, is prohibited, while in the other zones collection of basidiomata smaller than 3 cm in size is prohibited. *P. nebrodensis* is also protected by the park's regulations as well as by Regional Law No. 3 (February 1, 2006).

Moreover, subpopulations of *P. nebrodensis* fall within Natura 2000 sites, which are all included in the territory of the Madonie Mountains and more specifically in the following localities: ITA020004 "M. San Salvatore, M. Catarineci, Vallone Mandarini, humid environments, "ITA020016 "Monte Quacella, Monte dei Cervi, Pizzo. Carbonara, Monte Ferro, Pizzo Otiero", and ITA020020 "Evergreen oak forests of Geraci Siculo and Castelbuono".

One of the strategies for *ex situ* conservation, particularly for saprotrophic fungi whose mycelium can be easily reproduced in the laboratory, is that of cultivation

outside its habitat. This has considerable relevance in the case of fungal species at risk of extinction or closely confined to threatened habitats. In the Mediterranean region, the cultivation of *Pleurotus* species is diffused and represents *ca.* 10–20% of total mushroom production (Ferraro et al. 2022). The *ex situ* cultivation of *P. nebrodensis* is part of the project "PLEURÒN - Project for the cultivation of *Pleurotus nebrodensis* in a protected environment for food, medicinal and phytogenic purposes", recently approved (2023) by the Sicilian Administrative Region and aimed at cultivating the mushroom on a pilot scale and in a protected environment. The partnership consists of Consortia for Research, Universities and farms located in the Madonie territory. This ambitious project is based on previous positive experiences in growing *Pleurotus* mushrooms in Italy (Varese et al. 2011; Venturella et al. 2016). In particular, *P. nebrodensis* can be grown at different altitudes within semi-shaded tunnels. Moreover, cultivated *P. nebrodensis* basidiomata have the same organoleptic features as the wild basidiomata (Zervakis and Venturella 2002). Thus, *ex situ* cultivation will provide additional income to local farmers, with the possibility of selling the product at lower prices than those of mushrooms collected in the wild. Finally, *ex situ* cultivation would ensure a gradual reduction in pressure from the many seekers of the prized mushroom in nature.

In this survey we report the taxonomy, ecology, distribution, and potential applications of this important Sicilian endemic fungus. In addition, because the binomial "*Pleurotus nebrodensis*" is often misapplied, an attempt is made to help clarify the exact taxonomic placement of the mycelium marketed by a leading company selling mushroom-growing substrates under the name "*Pleurotus nebrodensis*".

Materials and methods

Collection, habitat details and morphological characters

Field research carried out in pastures of the Madonie mountains (N. Sicily, Italy) led to the collection of a white-colored mushroom on dead roots of *Prangos ferulacea* (L.) Lindl., a perennial herbaceous plant distributed in the Mediterranean Basin, the Bulgarian Black Sea coast, and the Caucasus. In accordance with the Prodrome of Vegetation in Italy (Biondi and Blasi 2005), *P. ferulacea* is part of the plant association no. 62.2.1 *Cerastio-Astragalion nebrodensis* Pignatti & Nimis ex Brullo 1984 in which xerophilous and basiphilous communities occurring on dolomitic substrates with more or less pronounced slopes, mainly on consolidated rocks and sometimes on rocky ridges, are present (Fig. 1). This plant association is distributed throughout the Madonie mountains in the supramediterranean thermotype.

Field excursions were carried out in the years 2022 and 2023 during the fruiting period of *P. nebrodensis* extending from late April to early June. The research localities fall in the area of Monte dei Cervi (1794 m), a mountain falling within the territories of Scillato and Polizzi Generosa, 37°52'45"N, 13°58'14"E (DMS), and Vallone Faguare a canyon located at 1,263 m a.s.l., 37°51'42"N, 14°03'54"E (DMS) in the

Figure 1. Pastures of *Prangos ferulacea* on the Madonie mountains of northern Sicily (Photo G. Venturella).

territory of Petralia Sottana (Madonie mountains). Based on the classification of Rivas-Martinez (1995), the bioclimatic characters of the area can be overall referred to the mesomediterranean (average temperature: 13–16 °C) and supramediterranean (average temperature: 8–13 °C) thermotypes, with ombrotype varying between subhumid (average rainfall: 600–1000 mm) and humid (average rainfall: >1000 mm).

Six fresh basidiomata of *P. nebrodensis* were collected and identified according to macroscopic characters (cap, flesh, lamellae, stipe, type of occurrence, color of spore prints, etc.). In addition, microscopic characters (basidiospores, basidia, cheilocystidia, hyphal system, hyphal wall, hyphae, and pellis) were observed at 40X-1000X (AmScope, Irvine, USA). The morphological examination was carried out according to Venturella et al. (2015).

The specimens (Fig. 2A) were dried at 40 °C in a 475 W stainless steel dryer (Mauro Valla, Borgotaro, Italy) and deposited in the Herbarium SAF of the Department of Agricultural, Food and Forest Sciences (SAAF 503) of the University of Palermo. The nomenclature of fungi follows Index Fungorum while the binomial of plants is referred to Euro + Med PlantBase (www.emplantbase.org).

Establishment of pure cultures

A piece of tissue from fresh basidiomata was placed on potato dextrose agar (PDA) in Petri dishes under aseptic conditions under a laminar flow hood. The Petri dishes were sealed with Parafilm and incubated at 25 ± 2 °C. The pure culture is kept in the Mycotheca of the Herbarium SAF (SAF 40) (Fig. 2B).

Figure 2. *Pleurotus nebrodensis* samples **a** Exsiccata of *P. nebrodensis* deposited in the Herbarium SAF of Palermo University **b** Pure culture of *P. nebrodensis* (Photo G. Mirabile).

Extraction of DNA, amplification, ITS sequencing and phylogenetic analysis

Twelve marketed cultivation bags (four strains, indicated as 1, G, 6, and 8, in three replicates), inoculated with *P. nebrodensis* mycelium, provided by Italmiko (Senise, Potenza), were analyzed in order to identify, by a molecular approach, the exact taxonomic identity of basidiomata. DNA was extracted from fresh basidiomata using the Extract-N-Amp™ kit (Sigma-Aldrich, St. Louis, USA) following the manufacturer's instructions. DNA purity and concentration were measured at 260/280 nm and 260/230 nm using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The Internal Transcribed Spacer (ITS) region of rDNA was amplified using ITS1F and ITS4 primers by polymerase chain reaction (PCR) in a total reaction volume of 20 µl (4 µl of extracted DNA, 1 µl of each primer at 10 µM ,10 µl of the Extract-N-Amp PCR reaction mix (Sigma-Aldrich, St. Louis, USA), and 4 µl of sterilized distilled water. The amplification was performed in a MultiGene OptiMax thermocycler (Labnet International Inc., Edison, USA) with the following parameters: 3 min of initial denaturation cycle at 94 °C; 35 cycles at 94 °C for 30 s; annealing stage at 55 °C for 30 s; elongation for 45 s at 72 °C and 10 min of final extension at 72 °C. PCR product was separated in 1.5% agarose gel by electrophoresis and detected under UV light. PCR product was purified using Exo I-SAP protocol (Applied Biosystems, Foster City, USA) and sent to BMR Genomics (Padova, Italy) for sequencing. In the sequencing reaction, only primer ITS1F was used. The obtained sequence was manually adjusted and compared with those in GenBank using the BLASTn tool (https://blast.ncbi.nlm.nih.gov).

The new sequences were deposited in GenBank. Sequences with 99–100% of similarity, as well as *P. eryngii* complex representative sequences from a previous ITS-phylogenetic study (Table 1, Zervakis et al. 2014) were obtained from GenBank and aligned with the isolated sequence obtained in this study. Alignments were performed using ClustalW software and manually adjusted, if necessary, using MEGA11. The Neighbour-Joining algorithm was used to generate the phylogenetic tree and the evolutionary distances were calculated based on Maximum Composite Likelihood. Bootstrap percentages were calculated from 1000 re-samplings.

Table 1. Strains of *Pleurotus* used for ITS-phylogenetic analysis. Those obtained in this study are in bold.

Taxon	Host	Geographic origin	Accession number
P. eryngii var. *eryngii*	*Eryngium* sp.	China	HM998840
P. eryngii var. *eryngii*	*Eryngium* sp.	Ukraine	HM998820
P. eryngii var. *eryngii*	*Eryngium* sp.	Italy	KF743828
P. eryngii var. *eryngii*	*Eryngium maritimum*	Greece	HM998811
P. eryngii	Commercial	China	HM998841
P. eryngii	Apiaceae	Iran	HM998833
P. eryngii	**Commercial**	**Italy**	OR681547
P. eryngii var. *elaeoselini*	*Laserpitium latifolium*	Italy	HM998827
P. eryngii var. *elaeoselini*	*Laserpitium siler*	Italy	HM998825
P. eryngii var. *elaeoselini*	*Elaeoselinum asclepium*	Italy	HM998819
P. eryngii var. *elaeoselini*	*Laserpitium latifolium*	Italy	KF743824
P. eryngii var. *ferulae*	*Ferula communis*	France	HM998808
P. eryngii var. *ferulae*	*Ferula communis*	Greece	HM998813
P. eryngii var. *ferulae*	*Ferula communis*	Greece	HM998814
P. eryngii var. *thapsiae*	*Thapsia garganica*	Italy	HM998815
P. eryngii subsp. *tuoliensis*	*Ferula* sp.	Iran	HM998836
P. eryngii subsp. *tuoliensis*	*Ferula sinkiangensis*	China	HM998839
P. eryngii subsp. *tuoliensis*	*Ferula sinkiangensis*	China	HM998842
P. nebrodensis	*Prangos ferulacea*	Greece	KF743821
P. nebrodensis	*Prangos ferulacea*	Italy	HM998818
P. nebrodensis	*Prangos ferulacea*	Greece	KF743820
P. nebrodensis	*Prangos ferulacea*	Greece	HM998826
P. nebrodensis	*Prangos ferulacea*	Italy	HM998816
P. nebrodensis	*Prangos ferulacea*	Italy	HM998832
P. nebrodensis	*Prangos ferulacea*	Italy	KF743830
P. nebrodensis	**Commercial**	**Italy**	OR681545
P. nebrodensis	**Commercial**	**Italy**	OR681546
P. nebrodensis	**Commercial**	**Italy**	OR681548
P. ferulaginis	Apiaceae	Iran	KF743829
P. ferulaginis	*Ferulago campestris*	Italy	KF743833
P. ferulaginis	*Ferulago campestris*	Italy	KF743826
P. ferulaginis	*Ferulago campestris*	Italy	KF743827

Results

Taxonomy

Pleurotus nebrodensis (Inzenga) Quél. was described under the binomial *Agaricus nebrodensis* by Giuseppe Inzenga (Inzenga 1863), one of the most eminent mycologists of the second half of the 19th century. Different binomials have been attributed to *P. nebrodensis* many of them have subsequently fallen into synonymy with *P. nebrodensis*. Saccardo (1915) considered *P. nebrodensis* as a variety of *Pleurotus eryngii* (DC.) Quél. while other authors report the binomials of *Agaricus fossulatus* Cooke (Aitchinson 1888) or *Dendrosarcus fossulatus* (Cooke) Kuntze (1898). The study of herbarium material deposited at the Muséum National d'Histoire Naturelle in Paris (Venturella 2000) revealed that the *exsiccatum* positioned in the center of the herbarium sheet (Fig. 3A) corresponds to *P. nebrodensis* and is

perfectly superimposable on the original drawing of Giuseppe Inzenga (Fig. 3B). The subsequent elucidation by Venturella et al. (2016) confirmed Inzenga's intuition that *P. nebrodensis* is a valid species and that the binomial *A. fossulatus* is to be referred to *P. nebrodensis* subsp. *fossulatus* (Cooke) Zervakis & Venturella, and is a related Asiatic taxon growing on *P. ferulacea.*

Morphological description

The basidiomata of *P. nebrodensis* (Fig. 4) are fleshy with a pileus 3.0–15.0 cm wide, applanate, uplifted, shallowly depressed, convex or conchate, light ivory to cream. The margin of the pileus is plane, incurved, uplifted with a surface entire or eroded, smooth. The cuticle is glossy or translucent, dry, smooth (glabrous) or becoming cracked. The color of the flesh is cream, with consistency hard-tough to turgid, color unchanging when cut, sulphur-yellow when dry, 1–2 mm thick at the margin and 1–4 cm thick at the center. The taste is mildly farinaceous. Lamellae 4–8 mm width, 2.5–7.5 cm length, annexed to decurrent, gill spacing sub-distant to close, moderately broad in thickness, light ivory, margin of gills smooth to eroded, face of gills waxy, lamellulae present, extending one-half to one-third the length of gills. Stipe 1.5–3 cm width, 2.5–4.5 cm length, terete in cross section, slightly tapered to tapered at the base. Consistency fibrous, flesh solid to stuffed. Stipe eccentrically or lateral attached to pileus, inserted in the root residues of *P. ferulacea*, basal tomentum and veil absent. The stipe surface is smooth, light ivory colored. The habit is solitary or connate. Basidiospore print light ivory to cream. Basidiospores 12.5–15.1(–18) × 5.2–6.1 µm, cream, asymmetrical, cylindrical to phaseoliform, smooth, hyaline, guttulate. Basidia 4-spored, with basidioles 40–50 × 10–11.5(–14) µm, sterigmata 3–4.5 µm. Cheilocystidia (leptocystidia) 50–60 × 6.2–7.5(–9) µm, clavate, apex mucronate to capitulate. Hyphal system monomitic. Hyphal wall thin. Hyphae septate with clamp connections. Specialized hyphae absent, no pigmentation. Pellis in two layers, 5–10 µm width.

Figure 3. *Pleurotus nebrodensis* **a** herbarium sheet deposited in PC showing a specimen of Sicilian provenance of *P. nebrodensis* (sub. *Agaricus nebrodensis* Inz.) in the center **b** the original drawing of the medium-sized basidiome of *P. nebrodensis* by Giuseppe Inzenga (1863).

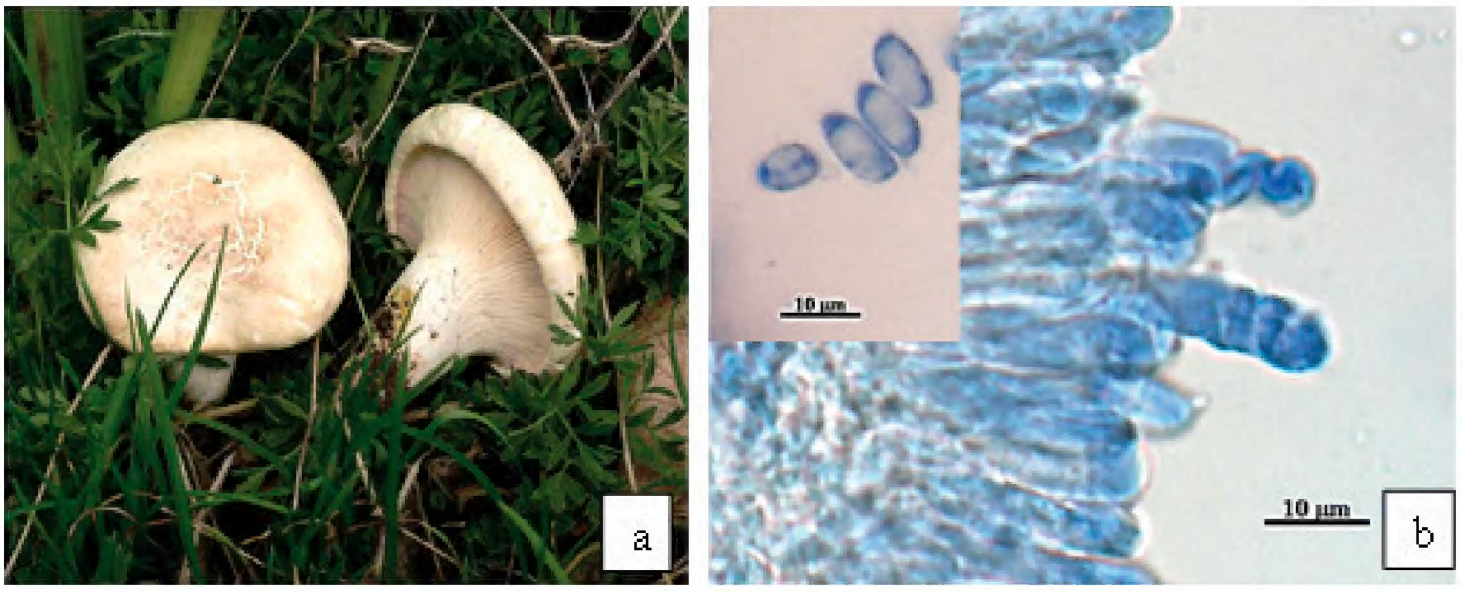

Figure 4. Basidiomata of *Pleurotus nebrodensis* **a** *In situ* (Photo G. Venturella) **b** microscopic features (basidia and spores) (Photo G. Mirabile).

Analysis of mycelium contained in commercial cultivation bags

In a separate publication, the commercial strains on the international market under the name "*Pleurotus nebrodensis*" were verified, and it was shown that the great part of them do not correspond to *P. nebrodensis* but should be referred to another taxon, i.e. *Pleurotus* subsp. *tuoliensis* (C.J. Mou) Zervakis & Venturella (Venturella et al. 2016). Fresh mushrooms morphologically similar to *P. nebrodensis* of uncertain taxonomic identity are still cultivated and marketed in Italy. Based on the certified source material belonging to true *P. nebrodensis*, preserved in the Herbarium SAF of the Department of Agricultural and Forestry Sciences (University of Palermo, Italy) we investigated 12 cultivation bags, labelled by the provider and inoculated with mycelium of "*P. nebrodensis*" and marketed by a leading company located in southern Italy that applied for certification.

Molecular and phylogenetic analysis of the twelve marketed cultivation bags (Fig. 5) showed that the mushrooms grown in bags labeled as 1 and 8 clustered with *P. nebrodensis*. The mushrooms obtained in the bags labeled with the letter "G" falls in the cluster *P. eryngii sensu stricto*, shown in Fig. 6.

Regarding the three replication of cultivation bags labeled as 6, two of them belongs to *P. nebrodensis* cluster (Fig. 6), while one replication, which presented a completely different mycelium morphology, resulted as *Irpex latemarginatus* (Durieu & Mont.) C.C. Chen & Sheng H. Wu, probably a contaminant.

Discussion

Italy, located in the center of the Mediterranean basin, is considered one of 34 global biodiversity hotspots (Mittermeier et al. 2011). Hotspots are key locations for biodiversity conservation because they have a high rate of endemic species. Sicily, due

Figure 5. Bag "G" producing *Pleurotus eryngii* mushrooms (left) and bag n. 1 producing the *P. nebrodensis* (right) (Photos G. Mirabile).

to its insularity and the topography of the land, is home to a rich animal, plant and fungal diversity. *Pleurotus nebrodensis* and its habitat are currently not protected by any international conservation rules. Consequently, there is an urgent need to raise awareness among policy makers and the scientific community to implement appropriate conservation actions and sustainable use towards this important natural resource. The regulation on mushroom picking by the Madonie Park Authority to limit the negative impact on *P. nebrodensis* fructification is often ignored by gatherers given the poor controls. *Ex situ* cultivation will reduce the pressure due to overharvesting in natural habitats and at the same time lower the cost of this prized mushroom, which is currently too high for the pockets of most consumers. Finally, given the environmental and economic value of the *P. nebrodensis* stand, it is desirable to encourage the involvement of citizens and an increase in public awareness for the protection and enhancement of *P. nebrodensis*. Citizen interest in this mushroom can also stimulated by the demonstrated medical application potential of *P. nebrodensis*. Different medicinal properties are attributed to the genus *Pleurotus* (Fr.) P. Kumm (Lesa et al. 2022). In recent years, data have been published on the antibacterial and antitumor properties of other *Pleurotus* species fruiting in the Mediterranean Basin. As regards *P. nebrodensis* from Sicily, it contains biologically active compounds that act in modulating the immune system and inhibiting the growth of cancer cells (Alam et al. 2011). Specifically, the water extract of *P. nebrodensis* is able to suppress proliferation of colon cancer cells without significant effects on proliferation of normal cells. It also has a potential application in contrasting the biofilm mode of growth of human pathogens. Cold water extracts were tested on human colon cancer cells (Fontana et al. 2014) with positive effects on antitumor activity and immunomodulation and increased natural killer cell activity.

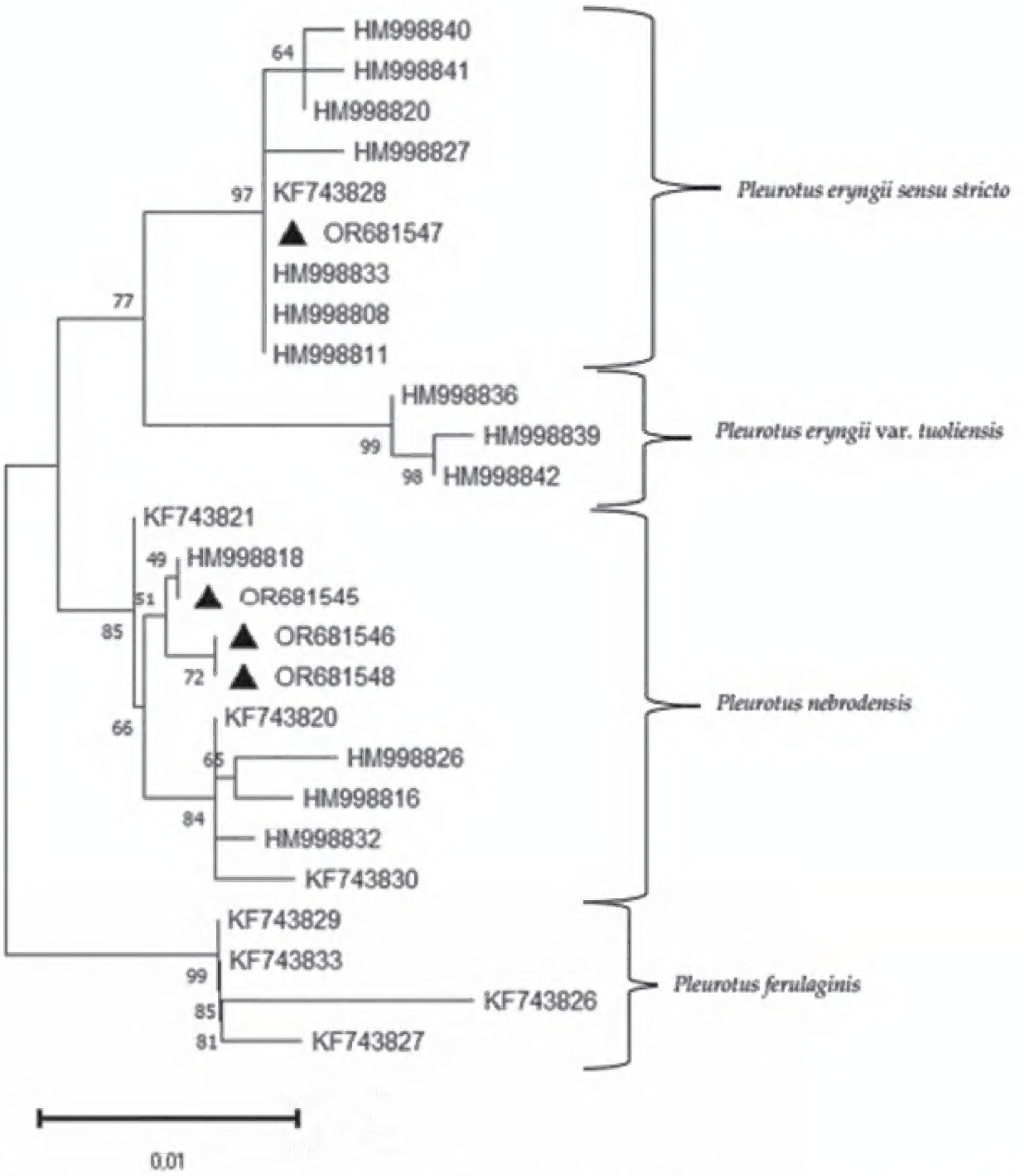

Figure 6. Phylogenetic tree obtained from the analysis of ITS1-5.8S-ITS2 sequences obtained from this study (labelled with black triangle) and additional sequences from NCBI.

The same extracts were also tested *in vivo* against medically relevant bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *S. aureus*, and *Escherichia coli*. These findings open interesting perspectives for the inclusion of *P. nebrodensis* among the most valuable mushroom-based products to be used in integrated medicine.

There is also a need to resolve the confusion that exists in the commercial exploitation of material bearing the name "*Pleurotus nebrodensis*" through accurate taxonomic identification in order to ensure that products on the market are of safe origin and genetic purity. This covers both the production of mushrooms for the food market and the supply of dried powders for the production of mushroom-based products. In the first case, there is a problem that can be traced to a practice long in use by mushroom hunters in the Madonie mountains of mixing basidiomata of *P. nebrodensis* with another morphologically similar white fungus named *P. eryngii* var. *elaeoselini*

Venturella, Zervakis & La Rocca (Fig. 7). However, this taxon is genetically separated from *P. nebrodensis* as it falls into the species complex of *P. eryngii* (Zervakis et al. 2014).

It has been recently demonstrated that the Italian market for mushroom-based products is characterized by products of dubious origin (Risoli et al. 2023). Thus, the future use of dried powders of *P. nebrodensis* in the production of mushroom-based products, one of the main targets of the above-mentioned project recently funded by the Sicilian Administrative Region, cannot disregard a careful review of all the genetic material sold by the companies that produce substrates and mushrooms in order to enable them to supply the market with the real "*P. nebrodensis*". In addition, based on the results that will emerge from the PLEURON project, we suggest not to allow the cultivation of species related to *P. nebrodensis* in the Madonie territory in order to avoid the risk of genetic mixing between congeneric species.

Figure 7. Basidiomata of *Pleurotus eryngii* var. *elaeoselini* are almost impossible to distinguish macroscopically from those of *P. nebrodensis*. (Photo G. Venturella).

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